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Chemical Constituents of the Water Extract of Beef

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1901

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The Chemical Constituents of the Water Extract of Beef

BY ARTHUR DONALDSON EMMETT

THESIS

FOR THE

Degree of Bachelor of Science in Chemistry

IN THE

COLLEGE OF SCIENCE

UNIVERSITY OF ILLINOIS

1901

1901
Emb

UNIVERSITY OF ILLINOIS

May 31st 1901

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

A. D. Emmett under Dr. Grindley

ENTITLED The Chemical constituents of the water extract of Beef

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE

OF B. S. in Chemistry

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C H E M I C A L C O N S T I T U E N T S O F T H E W A T E R E X T R A C T O F B E E F.

Food is that substance,either animal or vegetable,which when taken into the body will produce nourishment for the system. It has been found that in the natural or raw state,food contains more nourishing or renewing power than when in the cooked condition. This being true,it is evident that by the processes of cooking there is a loss of nutritive matter. Thus in the roasting of meat the loss goes to make up what we commonly call gravy and in the boiling of meat to make the broth or soup. It would then seem to be a very simple matter to determine,from the chemical standpoint,these losses,but such is by no means the case.

Some work has been done abroad upon this subject but very little in this country. In fact,the only work in this country that has been done in determining the losses involved in the cooking of meats,has been in this laboratory. Here the broiling or frying and boiling of meats have been studied. For the past two years,special stress has been put upon the latter process of cooking,using in almost every case lean beef. (1.) It was found that none of the nitrogenous constituents were volatilized during the heating and that the material lost in the cooking passed into the water to make up the broth. That the total amount lost varied directly with the quantity of the meat taken and the time used in heating or, in other words,the longer the time taken for cooking,the richer the broth.

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This last statement must be modified,for if the meat is thin or in small pieces the total loss is greater,for the same length of time, than if it were thick or in one piece. Hence it will be seen that the time used,the amount taken and the size of the piece are the three elements which vary the strength of the broth in any one process. It now appeared to be of interest and value to determine the composition of this soup or broth, and thence the exact quantity and quality of the material removed from the original meat. With this object in view,a complete analysis of the broth was made. The total solids,the ash,the fat,the albuminoid nitrogen and the nitrogen as flesh bases were determined separately. (2) The percentage loss of each was calculated to the total solids and their sum subtracted from 100%. By this,it was found that the percent of ash lost by the cooking of the meat was 56.4,of fat 17.7,of total nitrogen 5.2. The sum was 79.3% leaving 20.7% unaccounted for. In other words,it was found that the summation,in grams,of the ash,the fat,the albuminoid nitrogen and that of the flesh bases did not equal that of the total solids. Such results were entirely unexpected. However,repeated experiments gave analogous results. It was thought that such a condition of things might be due,at least in part,to water of crystallization,to the method,to the use of a wrong nitrogen factor for flesh bases and to the presence of non-nitrogenous matter.

The first to take up the study of meat broth was A.Vogel in 1884. (3) He found that by placing the meat in cold water and then raising the temperature to the desired degree,that a richer soup

could be obtained than by having the temperature at the boiling point before adding the meat. The high heat coagulated the albumin upon the surface and thus inclosed the nutrient portion which would otherwise have been removed. (4) In 1893,Fr.Nothwang experimented upon the losses involved in the boiling and steaming of fresh and salted meats. He determined the total solid matter separated from the meat,and the salts and total nitrogen in the clear broth. In an article,(5) by C.D. Woods,upon meat,he quotes the result of a german experiment as follows:a pound of beef and seven ounces of veal bone were used,the broth from which contained 95.2% of water,1.2 % of protein,1.5 % of fat,13 % of ash and 1.8 % of extractives. In 1897,A.Gautier,a Frenchman,took up the subject and published an article upon,"The Value of the Importation of Refrigerated Meats." (6) In this,he gave the analysis of a beef broth,showing in a most detailed manner its composition. He claimed that the total dry substance in a liter of broth was composed of 10.9 % creatin and other bases,8 % inosite,glycogen and other reducing bodies,24 % ash,14 % extractives and 42.9 % other nitrogenous substances. Here,none of the constituents were found by difference. Nothing further was done upon the analysis of broths until 1898,(7) when the subject was taken up in our own laboratory by Grindley and McCormack. They found that one sixteenth of the nitrogen,one eighth of the fat and one half of the ash were extracted from the meat in the cooking. In 1899,(8) Grindley and Porter continued the work and found

results agreeing very closely with those given above as regards the amount of nutrients removed. They covered almost the same ground as Gautier. There were no determinations made for inosite,glycogen and other reducing substances. In the following table the results of the two experiments are given.

Constituents in the Beef Broth.	Grindley and Porter	Gautier.
	%	%
Creatin and other bases.----------	27.7	10.9
Inosite,glycogen and other reducing substances.----------	----------	8.0
Mineral matter----------	23.8	24.0
Extractives----------	14.4	14.0
Albumins,peptones and gelatenoids etc.-----	13.6	42.9

By comparison,it will be seen that the greatest difference between the two experiments lies in the percentage of the nitrogenous bodies. The various methods of cooking may account for part,if not all, of this difference. Gautier took one kilogram of meat and cooked it in 2500 c.c. of distilled water for a "long time". He stated,that when salt was added to the meat,that after cooking for from eight to ten hours,there was a partial peptonisation and solution of the glues. Hence this could account in part,if salt were used,for his high percentage of nitrogen,the gelatin portion of which,alone,is 1.5 % more than Grindley and Porter's total proteid nitrogen. Again, he may have taken a higher nitrogen factor for flesh bases than the 3.12,which was used here. In either case,we are not justified in

comparing our results with any of these investigators. They have given no definite method and,in most every case,they have found some one constituent by difference which would balance one error by a far greater one.

Closely allied to beef broth is meat extract. It is composed of almost the same constituents as the broth and is made by taking a cold water extract of the fresh meat,boiling the clear liquid,then refiltering and finally evaporating to the proper consistency. It was thought that the results which have been obtained in the investigations upon meat extracts would probably throw some light upon the problem which has arisen in connection with the study of meat broths. The first to analyze meat extracts were the German chemists:Liebig,König and Stutzer. (9).(10) Their object was mainly to determine the physiological value of the substance and the estimation of its nutritive constituents,of which reference will be made later. They did not go as far into the analysis as some of the later investigators,having taken many of their substances by difference. (11) In 1899,Mr. Thomas Macfarlane,of the Inland Revenue Department,Canada,published a very able paper upon meat extracts. He found that there was 26.12 % of organic constituents,whose identity was not known. This probably corresponds to the material in meat broth,which is not capable of being determined by the ordinary methods of analysis. His method of procedure was to make a 94 % alcoholic extract and then to analyze it. He found that there was 56.07 % removed from the sample. Of this,

the ash was 21.65 % and the organic matter 34.42 %. The nitrogen content, calculated as flesh-bases, was 8.30 %. When this is deducted from the percentage of organic matter, we have 26.12 % of organic non-nitrogenous material. Mr. Macfarlane confirmed his statement by the results of his assistant, McGill. The following table gives a few results. Group number one contains analyses of beef extracts and group number two that of a beef juice.

Table No.1.

Constituents.	Group No.1.			Group No.2.
	No.1.	No.9.	No.10.	No.15.
	%	%	%	%
Water----------	21.900	20.800	25.200	54.600
Ash----------	22.200	17.600	33.000	11.100
Fat----------	1.070	1:020	0.230	0.940
Insoluble proteids------	3.575	3.431	0.813	.....
Proteids ppt'd by bromine	6.290	5.425	9.456	2.006
Flesh bases----------	18.876	24.845	8.867	8.867
Undetermined by diff.---	26.089	26.879	22.434	22.487
Total----------	100.000	100.000	100.000	100.000

Mr. Mcfarlane says: "From this, it will be seen that, not only does the percentage of flesh bases, determined by the method which Mr. McGill describes, correspond closely with that obtained by extracting with 94 % alcohol, but that the presence of a very large percentage of

undetermined constituents is confirmed. These are mentioned by Allen as non-nitrogenous extractive matters and are probably carbohydrates of some description derived from the glycogen contained in the original meat."

Nothing further has been found in direct connection with this work,with the exception of a recent and brief notice by Bigelow,(12) in which he indicates that he is working along this line. Reference will be made to this later.

As previously stated,an error might be due to the nitrogen factor. (13) It was Stützer,who after studying the different forms of nitrogen,found that in the determination of the flesh-bases that the nitrogen ought to be multiplied by some factor less than Hehner's,which is 6.25. He finally settled upon 3.12,which while he knew it was too low,he considered it far better than the higher one. Hehner however,preferred to keep his,beleiving that it made the non-nitrogenous extractives lower and the nitrogen as flesh bases higher,therefore obtaining the more accurate result. Gautier may have used this latter factor in his work. In Bigelow's notice, he stated that bromine did not precipitate all the proteids. If this be true then we are led to beleive that Hehner was,at least partially,right in his theory. For,if some of the proteids are left in solution,it is evident that we must not calculate them as flesh bases by using the factor 3.12. On the other hand,we have found,by using the Hehner factor on all the flesh bases,that in only a few cases did the total summation approximate to 100 %. In other words,by taking the sum of all the constituents and cal-

culating the total nitrogen by multiplying it by Hehner's factor, 6.25,we obtained results as just stated. In the majority of cases, they were below,but in some above the correct percentage. Where the latter case occured,it will be noted,by comparing the results in the following tables,that the variation is due to the extractive matter more than to the proteids. In table number two the nitrogen as flesh bases is calculated according to Stützer,and in number three according to Hehner. The samples have all been prepared by the same process and were obtained from meat of as near the same kinds as possible. Laboratory numbers 777a,892,894 and 1146 are to be compared with 781a,809,1147 and 1158 respectively.

Table No.2.

Lab.No	Protein (Nx6.25)	Flesh Base. (Nx3.12)	Extractive or Fat Matter.	Ash.	Total.	Difference from 100	Diff. between 2samples
	%	%	%	%	%	%	%
777a	12.89	27.31	13.61	24.60	78.41	-21.59	.....
781a	12.54	25.44	1.87	23.04	62.89	-37.11	-15.52
892	6.12	30.62	9.06	25.74	71.54	-28.46	--...
809	5.82	29.20	2.12	24.84	61.98	-38.02	- 9.56
894	4.28	30.54	8.02	25.02	67.86	-32.14	.....
1147	4.97	30.41	2.22	24.27	61.87	-38.87	- 6.73
1146	5.47	30.72	9.57	22.67	68.43	-31.57	.....
1158	4.80	29.01	1.05	21.82	56.68.	-44.32	-12.75

Table No.3.

Lab.No.	Protein (Nx6.25)	Flesh Base (Nx6.25)	Extractive or Fat Matter.	Ash	Total	Diff. from 100	Diff minus diff bet. Fat of two samples.
	%	%	%	%	%	%	%
777a	12.89	54.63	13.61	24.60	105.73	+5.73	-6.01
781a	12.54	50.89	1.87	23.04	88.34	+11.66	.....
892	6.12	61.25	9.06	25.74	102.17	+2.17	-4.77
809	5.82	58.41	2.12	24.84	91.19	-8.81	.....
894	4.28	61.09	8.02	25.02	98.42	-1.58	-7.38
1147	4.97	60.83	2.22	24.27	92.30	-7.70	.....
1146	5.47	60.45	9.57	22.67	99.16	-0.84	-9.36
1158	4.80	58.03	1.05	21.82	85.70	+14.30	.....

It is apparent from these tables that,if Mr.Bigelow is correct,neither of the nitrogen factors will suffice;that from the great variations in the calculations and the fact that the extractive matter appears to be the chief cause,there must be something besides the nitrogen factor question that goes to help make up this deficiency. It was thought that water held mechanically in the total solids might cause some appreciable error. But we found that this was not true. We first dried the samples to a constant weight in a water oven at 100° C,then in a glycerine oven at 105°C and finally in a hot air oven at 110° C. The variation in the weights,by the three heatings was so small,that it was discarded

as due to slight decomposition of the material itself. The following table needs no explanation.

Table No.4.

Temp.at 100°C:	Temperature at 105°C.			Temperature at 110°C.		
Weight of Solids.	Weight of Solids:	Loss in Grams.:	Percentage loss:	Weight of Solids:	Loss in Grams:	Percentage Loss.
.4707	.4581	.0126	2.67	.4549	.0158	3.35
.4703	.4573	.0130	2.76	.4543	.0160	3.40

We next turned our attention to the methods of analysis. A number of analyses of meat broth were made,using practically the same method as Mr. Porter. Our results showed the same facts as those of Grindley and Porter,that is,that there is apparently a considerable quantity of organic material in broths which is not estimated by the ordinary method of food analysis. However, to be more certain,the method was tried by a third person. Exactly the same conclusions were reached. Of twenty-nine of these experiments(14) the average is as follows: total solids,22.1 grams; summation of all the determined constituents,14.5 grams; other substances by difference,7.6 grams,and the per cent of these calculated to the total solids,34.2. In connection with this study Mr. Macfarlane's article upon meat extracts was brought to our notice. Hence,we feel justified in making the assertion that the

method of analysis is approximately correct, and can in no way be the cause of the peculiar results which were found. In table number five, is arranged a portion of the data of the three above mentioned experiments.

Table No.5.

Lab. No.	Total Solids by Direct Det. grams	Protein, (Nx6.25) grams	Flesh Bases (Nx3.12) grams	Fat grams	Ash grams	Total by summation grams	Other subs. by Diff. grams	Per cent Diff. %
Experiment No.1.(Porter.)								
777a.	20.388	2.628	5.568	2.776	5.016	15.988	4.400	21.59
779a.	20.140	2.964	5.480	2.800	4.832	16.076	4.064	20.16
781a.	17.316	2.176	4.416	0.332	4.010	10.934	6.382	37.11
809a.	18.416	1.072	5.376	0.392	4.576	11.416	7.000.	38.02
Experiment No.2.(Emmett.)								
892.	21.230	1.300	6.501	1.924	5.464	15.189	6.041	28.46
893	25.356	2.436	7.375	2.160	6.140	18.111	7.245	28.65
894	12.668	0.542	3.869	1.016	3.170	8.597	4.071	32.14
895	51.128	2.478	15.305	3.988	2.276	34.048	17.086	33.42

Experiment No.3.(Grindley.)

1146	15.983	0.875	4.910	0.143	3.623	9.551	6.432	31.57
1147	41.907	2.084	12.745	0.091	10.170	25.090	16.817	38.87
1158	22.085	1.059	6.408	0.232	4.818	12.517	9.568	44.32
1160	14.014	1.144	3.864	0.214	2.996	8.218	5.796	41.36

In our opinion,there remains but one more source for the error. Since it is not due to the method,nor to the presence of water of crystallization and,if at all,only in part to the nitrogen factor for flesh-bases,then,since the amount of extractive matter seems to vary the percentage,it must be due to the presence of organic non-nitrogenous matter or to organic matter containing only a small percentage of nitrogen. As further evidence of this,(15) Miss K.I.Williams,who has been working upon the composition of fish flesh,found other reducing bodies than those normally supposed to be present and hence led us to beleive more firmly,that there were perhaps others in meat broth.

It is a well known fact that meat broth contains ingredients which are nutritious and also stimulating.(16) Liebig and Thudichum proved very conclusively that this latter property,in meat extracts,was due to the presence of creatin,creatinine,lactic acid and the potassium salts. These also furnish the flavoring property. It is therefore quite likely that these same bodies aid in effecting broth in a similar manner. However,it must

be stated,that the form in which these substances occur,after the cooking,is not necessarily the same as in the fresh meat or the extract. The creatin may,for example,be changed into creatinine. Hence in determining the exact losses in cooking,an error might be introduced by not taking this fact into consideration. In our work,we have attempted to take this decomposition or changing element into account and investigate it.

Broth or soup,from the boiling of beef,is composed of mineral matter,water,fat,nitrogenous and non-nitrogenous bodies. It has previously been stated that the strength of the solution varied according to the method of procedure. (17) Srohmer says, that from thirty to forty percent of the ingredients pass into the water. (18) Andrews,that not more than twenty percent,while it has been found,in this laboratory that from fifteen to forty per cent are removed from the meat. This percentage loss is found by weighing the meat before and after cooking and then calculating the difference as loss. Thus water,fat,ash and the nutrients are included under the one head.

The fat or ether extractive matter consists of the compounds formed by the higher fatty acids and the unsaturated acid series with glycerine. Also of other compounds not known. The ash or mineral matter is made up largely of potassium and sodium salts,together with phosphates. The nitrogenous material is composed of proteid and albuminoid substances. The distinction between the two is so difficult that for ordinary purposes they are classed under the former name,proteids. These are,according to

Wroblewski,bodies which when treated with an acid yield ammonia, nitrogenous bases and amidocompounds. They are divided as to their chemical and physical behavior into albumins,proteoses,gelatinoids, globulins,peptones and the flesh bases,-creatin and creatinine. Albumins are soluble in water,coagulated by heat,precipitated by lead acetate,mercuric and stannous chloride. Proteoses are soluble in water,not coagulated by heat,precipitated by neutral salts,nitric acid,alcohol,bromine and mercuric chloride. Gelatinoids are soluble in hot water but insoluble in cold water,alcohol,ether and chloroform,not precipitated by lead acetate or acetic acid but by mercuric chloride,bromine and tannin. Peptones are soluble in water, not coagulated by heat,not precipitated by neutral salts or zinc sulphate but by tannin and alcohol. Globulins are insoluble in water,coagulated by heat and precipitated by neutral salts. Flesh bases are the proteid bodies which contain a higher percentage of nitrogen and no sulphur. Creatin is readily soluble in hot water, insoluble in absolute alcohol and ether,changed into creatinine by heat and by heating with a mineral acid,not precipitated by mercuric chloride,silver nitrate and phosphotungstic acid. Creatinine is the anhydride of creatin. It is easily soluble in hot water and alcohol,insoluble in absolute alcohol,forms compounds with acids being a strong base,is precipitated by basic lead acetate,zinc chloride,mercuric chloride and phospho-tungstic acid. (19) In addition to these nitrogenous compounds,we may add inosinic acid and the lecithins. When the former is boiled,it decomposes into hypoxanthene,trioxyvaleric and phosphoric acids. It is formed from in-

osite. Lecithins are phosphorus compounds. They are soluble in water,quite so in ether and alcohol. Upon saponification,they break up into choline,glycerin-phosphoric acid,stearic and palmatic acids. Glycerin-phosphoric acid is a syrupy liquid,soluble in water,ether and alcohol. Its salts are all insoluble in alcohol excepting that of lead.

Besides these nitrogenous substances,we have the non-nitrogenous consisting of lactic acid,glycogen,inosite and other reducing bodies. Glycogen is easily soluble in water,insoluble in ether, absolute alcohol,precipitated by sixty percent alcohol,neutral salts,basic lead acetate,inverted by mineral acids and reduces Fehling's solution. Inosite is readily soluble in hot water and dilute acetic acid,insoluble in absolute alcohol and ether,does not reduce Fehling's solution but is precipitated by basic lead acetate. It decomposes into propionic,butyric and ordinary lactic acids.

We have now considered in detail the composition and chemical properties of the various known ingredients of beef broth. The next step will be to study their removal and estimation. As far as we have been able to learn,there is no method outlined whereby all the nitrogenous constituents can be completely separated from the non-nitrogenous. Hence to attempt to do this,we must resort to what others have done upon the separation of one or more of the individual compounds.

Perhaps,the first method for the separation of proteids from their substances,was that of coagulation by heat. It was found

that upon heating a solution containing all the proteids that a precipitation or coagulation took place at certain temperatures. (20) On further investigation,albumin was found to separate at 73°C and globuline at from 56°C to 75°C. (21) In 1885,O.Hehner used alcohol of different strengths to bring down different proteid compounds.(22)In 1893,Kemmerick found that 60% alcohol precipitated gelatin and 80 % the proteoses. Gamgee also adds that 100 % renders creatin,creatinines and peptones insoluble. In the latter case, an excess must be used. J.W.Mallet states that the use of alcohol is very incomplete for separating the different forms of nitrogen,as some of them are carried down mechanically with the others and it is almost impossible to wash them out. For our purpose, this would only add to the value of the reagent. (23)In 1896,Mr. Schjering discovered that by adding to solutions of different proteids,metals of the same series,that the amount precipitated in each case was the same. Then with the aid of other investigators, he experiment^ed using metals of different series and obtained quantities which were not alike. He used the following salts: stannous chloride,lead acetate,mercuric chloride,ferric acetate,magnisium sulphate and uranium acetate. Stannous chloride precipitated the albumins; lead acetate and mercuric chloride,albumins and denuclein; ferric acetate,albumins,denuclein and proteoses,magnesium sulphate albumins and proteoses,and uranium acetate,albumins,denuclein,proteoses and peptones. He now combined these in such a manner that by treating the filtrate from one by a second salt,then treating its filtrate in turn by a third salt and so on,that he could sep-

arate the albumins,the denuclein,the proteoses and finally the peptones from each other. (24)In close connection with this process is the one of saturating the solution of the proteids with salts. The most commonly used salts are zinc,magnesium and ammonium sulphates and sodium chloride. It is supposed that there is no such combination as that of the salt uniting with the compound as in the previous case but that the water which holds the compounds in solution is taken up by the salts and thus separating out the proteids in their uncombined state. Zinc sulphate removes albumin,proteoses,globulin and creatine but not peptones. Ammonium sulphate has the same effect,with the exception of creatin,as the zinc salt but it has a disadvantage in that it is difficult to remove all the ammonia,thus introducing an error. Sodium chloride brings down the proteoses,gelatinoids,globulins when in neutral solution. The next to take up this subject were Redeal and Stewart in 1897. (25) They used chlorine gas as their reagent. They weighed the dried precipitate,caused by saturating the solution,and used as their factors 0.78. It was claimed,that all the proteids except the flesh bases were thrown down. In the same year, bromine was tried in place of the chlorine. Allen aided in this and modified the method by determining the nitrogen in the wet precipitates. The results were the same as in the other case.(26) H.W.Wiley modified the method still further,applying it to meat products so that it has been used considerably of late.(27)Another method is that of Stützer. It is the use of cupric hydroxid. The author stated that peptones were incompletely precipitat-

and Mallet says, that some of the simpler amido-acids form salts with the hydroxid, which are very insoluble and hence remain behind with the proteids copper salt. The use of the phosphotungstic acid reagent has become prominent of late. (28)Mallet, upon finding that so many of the above methods and others did not account for the amid nitrogen compounds such as creatin, tried this reagent. He first separated out all the other compounds by heating the solution to 90°C in an acetic acid media. By this, he removed all except peptones, which were gotten rid of by means of tannin. Now the phospho-tungstic acid was added to this final filtrate until the precipitation was complete. The nitrogen determination on this, subtracted from the total, gave his desired value. Besides these methods for separating proteid bodies, there are others which are of no practical use. According to Mallet, they are dialysis, nitrous acid, potassium permanganate in the presence of free acid or alkali, sodium hypobromite, phenol, trichloracetic acid, formic aldehyde and hydrazoic acid. Recently, Mr. Bigelow(29)stated that he had tested, as to their accuracy the following methods for the separation and estimation of the nitrogenous constituents of foods and food products; Bromine, zinc sulphate, tannin and phosphotungstic acid. Also bromine in zinc sulphate filtrate. He found that bromine, as previously stated, precipitated only a small portion of the proteid matter and by the use of zinc sulphate with bromine, the result was more satisfactory.

We shall now consider the non-nitrogenous compounds-glyco-

gen and inosite. (30) Abel's method,for separating the non-nitrogenous constituents from solutions containing them,consisted in boiling the solution with potassium hydroxide and then almost neutralizing it with hydro-chloric acid. Now,he added to this, a solution of zinc chloride. The precipitate formed contained the non-proteid body.(31) Young separated out the compound by saturating the solution with ammonium or sodium sulphate at 33°C. (32) Haywood's method seems to give the best results. He made the solution slightly acid with hydro-chloric acid,then added two cubic centimeters of the dilute acid(1-5) and ten C.C.of Mäyer's reagent-the double iodide of potassium and mercury. This alternate treatment was continued until the filtrate showed the test for completeness. By this,the proteid matter was thrown down. Now to the filtrate,he added a few drops of concentrated hydrochloric acid and three times the volume of the solution of 95% alcohol. After three hours,he filtered, washed with alcohol and ether. ~~and washed~~. Dried the precipitate at 110°C and weighed it. Next, he extracted this weighed residue with water and calculated the loss as glycogen. (33) For inosite,Maquenne treated the solution with milk of lime,then added to the filtrate lead acetate. This left the inosite in the solution. To this,he added ammonical basic lead acetate and filtered. Removed excess of lead by hydrogen sulphide and evaporated the clear liquid to a syrupy consistency. Added 8% nitric acid and boiled,then added alcohol and ether. The inosite crystallized out. In determining the reducing

substances in fish Miss Williams did as follows:removed the fat by means of benzene,-then inverted the residue by adding ten c.c. of dilute hydrochloric acid and 100 c.c.of water,and heated on a water bath for three hours. She filtered this and added basic lead acetate to the filtrate,and again filtered. To remove the excess of lead,she used sulphur di-oxide. The remaining filtrate was evaporated to 100 c.c. and washed alumina added until it no longer dissolved. The clear filtrate from this was evaporated to dryness and then taken up with boiling alcohol,filtered and the residue dissolved in water. Then this was filtered through animal charcoal and titrated with Fehling's solution.

All the practical methods for the separation and estimation of both the nitrogenous and non-nitrogenous constituents in beef broth have now been considered. Below are recorded the methods which were used and the results obtained in separating,identifying and studying certain organic constituents of beef broth which heretofore have not been investigated.

Preliminary Methods used in Analyses.

Total Solid Determination.

In determining the total solids of all the liquids,an aliquot portion was measured out by means of a pipette into a weighed platinum dish and evaporated to dryness upon a water bath. It was then placed in a water oven and heated at 100°C until constant in weight.

Ash Determination.

The weighed total solids were taken and carefully ignited until all the organic matter was oxidized. In a few cases where it was difficult to obtain a white ash the dish was cooled and pure ammonium nitrate then added in small portions, after which the heat was again applied to the dish. No corrections were made for the volitization of sodium and potassium chlorides.

Nitrogen Determination.

The modified Kjehldahl process was used throughout with two slight modifications, when necessary. If the salt or solution contained mercury, we added none, and took the precaution to add enough potassium sulphide to precipitate all the mercury that was present. Secondly, if alcohol or ether were in the solution, water was added and the volatile liquid boiled off. The flask was then cooled and the process continued as in other cases. Standard hydrochloric acid and ammonia were used with one half cubic centimeter of lacmoid as indicator.

Albuminoid Nitrogen Determination.

An aliquot portion of the solution in question was taken and diluted with 200 c.c. of pure ammonia free water. Ten cubic centimeters of dilute hydrochloric acid were added and then bromine in small quantities while shaking vigorously. A globule of

the bromine was always kept present. When saturated,the solution was allowed to stand for at least twelve hours,after which it was filtered through a double paper filter previously washed with bromine water. The precipitate,together with the paper,was put in a Kjeldahl flask and nitrogen determined upon it in the usual manner.

Fat Determination.

The paper coil method was used. An aliquot portion of the clear solution was measured out by means of a pipette into a small beaker. The solution was then spread by means of the pipette over the coil as rapidly as possible and yet without crowding. The last portion was removed from the beaker by using one end of the paper coil. The rolled coil was now dried in a water oven,after which it was placed in a Söhlet tube and attached to a Hopkin's condensing apparatus. Here,it was extracted for twenty-four hours or more,according to the solution or material used. The extracted residue in the flask was weighed and saved for analysis.

Preparation of Broth.

A piece of lean beef of about one kilogram in weight was used in each cooking. All the visible fat,bone and grissle were removed. The meat was then placed in a vessel containing boiling ammonia free water of sufficient quantity to completely cover the

piece of meat. Glass rods were put on the bottom of the vessel to keep the meat from burning. No salt was added. The lid was put on and the boiling continued for ten minutes. The temperature was then allowed to fall to about 80-85°C. It was kept at this point for three hours when the meat was considered to be properly cooked. It was removed and placed in a glass jar to drain. In the meantime, the soup was cooled by running water in order to solidify the greater portion of the fat. This,together with the separated particles,was filtered through a weighed cheese cloth,filter. The vessel was thouroughly washed with distilled water and all the fat, remaining on the sides of the vessel removed to the filter by means of a spatula. The drippings from the meat were now added to the main portion. During this portion of the work,the temperature was kept below that of the melting point of the fat. The filtrate was now evaporated to a little less than 2000 c.c.,when it was cooled and made up to exactly that amount. Having thouroughly mixed the liquid,duplicate portions of 200 c.c.were measured off and filtered,through weighed paper filters,into 250 c.c. measuring flasks. The papers were washed and the volume made up to the proper mark. Total nitrogen,albuminoid nitrogen,total solid,ash and fat determinations were made upon this solution and the results calculated to the total volume. The remainder of the 2000 c.c. was filtered through a paper filter and used in further work.

Analytical Work.

The broth,for this investigation,was obtained in the manner

just described. In each case,the combined broths from the cooking of about twenty kilograms of beef were used. This is equivalent to 32000 c.c. of broth,which when evaporated to one liter gives a product of a rich deep wine color and of a high specific gravity. It was almost of a syrupy consistency,and it was expected that, upon standing,crystals of some sort,probably creatin,would separate. But after waiting ten days none were formed.

By the heating at 100°C,it was expected that complete coagulation of all the albumin and globulin would be effected. A flocculent brownish precipitate came down,which was filtered off and the clear filtrate made up to one liter. If this treatment was successful,the following known ingredients were left in the solution:proteoses,gelatinoids,peptones,creatin,creatinine,glycogen,inosite,other reducing substances,the acids,mineral matter and some fat.

Analysis of this filtrate was made by determining the albuminoid nitrogen, nitrogen as flesh-bases,total solids,ash and fat. The remaining portion of the solution was evaporated to almost a pasty condition and,while hot,transfered to a large flask, then extracted with 95 per cent alcohol until a test portion of the filtrate gave little or no residue upon evaporization. It was hoped that by this means,a second separation had been accomplished,on account of the insolibility of some of the constituents in alcohol of over ninty per cent. When the first portion of the alcohol was added,a cloudy precipitate appeared but upon increas-

ing the amount,it was changed to a dark brown flocculent one and finally to a sticky,semigranular mass. The supernant alcohol liquid was decanted through a filter and the residue repeatidly washed with ninety five per cent alcohol. The filtrate from this was distilled until its volume was reduced to about a liter,when it was cooled,refiltered and made up to a definite amount. One cubic centimeter of formalin was added before completing the volume. According to Lipiere,(34) no action takes place with this reagent at ordinary temperature,other than to prevent decomposition. The following ingredients have now been removed,either wholly or in part,proteoses,gelatenoids,glycogen,inosite,peptones and mineral matter. Some of the peptones are soluble in ninty five per cent alcohol,hence some of them were not removed. Likewise,some of the mineral matter as phosphates,sulphates and chlorides were left in solution. Then in this extract there were left from the original ingredients of the broth creatinine,creatine,the acids,some peptones,mineral matter and perhaps reducing substances. Analysis of this filtrate was made and the results are here given for the total amount:

Total solids--------	229.76 grs----------	
Ash----------	30.68 " --------	13.35 %
Nitrogen by bromine--	11.12 " --------	4.84 %
Nit.as flesh bases---	83.02 " --------	36.14 %
Other substances by difference-------	104.94 " --------	45.67 %

The next step ~~was taken~~ was that of removing the phosphates which were thought to interfere with the constancy in weights of the total solid determination. To do this,the alcoholic filtrate was tritrated to the neutral point with a standard solution of barium hydroxide,using litmus paper as indicator. By this reagent, the lactic acid was thought to also have been separated. If the total amount of barium used in the tritration was calculated to the lactate,the percentage of the acid would be 11.28 or 25.92 grams. This is .11 percent of the original meat which according to Mitchell(35),ought to vary from .1 to 1 percent. The end reaction was very hard to determine in this case and the only apparent remedy was to filter the solution through animal charcoal,a process which was not deemed of sufficient importance to compensate for the material necessary for its accomplishemnt. The titrated solution was allowed to stand twenty-four hours,with occasional stirring,after which it was filtered and the filtrate then evaporated to a small volume. The insoluble residue,which was a dark brown crystalline substance,was dissolved in boiling water and dilute hydrochloric acid. Analysis of this is as follows:

Total solids---------- -4.00 grs.

Ash----------3.21 grs.

Nit.as flesh-bases-------0.02 ".

From this it will be seen that most of the material precipitated by the barium reagent was inorganic matter.

Having now supposeidly gotten rid of the phosphates and lactates,attention was again turned to the separation of the most important of the remaining nitrogenous constituents,creatin and creatines. A review of their specific properties may not be amiss. Creatin,it is claimed,occurs in meat in greater proportion than creatinine. Its formula is $C_4H_9N_3O_2$. The percentage of nitrogen in it is 34.71. The crystals are transparent,colorless,oblique, rhombic columns. At 100° C.,they lose their water of crystallization. According to Mallet(36),one hundred grams of 90 % alcohol dissolves 0.018 grs.of the material. Mineral acids,upon heating, change creatin into the anhydrid creatinine-$C_4H_7N_3O$.. Creatinine is a very strong base,being alkaline to litmus. At 19°C,it is twenty-five imes as soluble in 90 % alcohol as creatin,hence it ought to be in greater proportion in the above alcoholic extract (37). It forms double salts with zinc chloride,mercuric chloride and silver nitrate. According to Voitessior(38),the zinc salt does not completely precipitate the creatinine. Schjerning states(39), that the mercury salt precipitates 97.2 % of the total nitrogen in blood serum,30-2 % in Wittes peptones and 15.8 % in Liebigs meat extract. Johnson gives (40),for the double mercuric chloride salt,the formula $4(C_4H_7N_3O.HCl.HgCl).3HgCl_2$. It contains 7.38% of nitrogen,61.53 % of mercury and 71.38 % of mercuric sulphide. This salt is decomposed by hydrogen sulphide into creatinine,mercuric sulphide,and hydrochloric acid.

The evaporated filtrate from the baryta-water,while hot,was treated with a saturated solution of mercuric chloride. A light yellowish,flocculent precipitate began to form at once. Upon adding more of the reagent,the quantity of the precipitate was increased. It became darker in color and finally granular. After standing for twenty-four hours with occassional stirring,it was filtered. It was noticed that quite a good deal of the coloring matter had been removed from the liquid by the process. The clear filtrate was evaporated upon the water bath to a small volume and a second precipitate was found to have been formed. From this, it is apparent that the mercury salt was more insoluble in hot than in cold solutions. The second salt was filtered off while the liquid was still hot. These two double mercury salts were considered to be practically the same as those which Johnson mentioned in his work upon urine,but analysis of each was made.

The first was found to contain:

		Theoretical(Johnson).
Nitrogen----------	6.89 %-	7.38 %
Mercuric sulphide---	65.95 %-	71.38 %
Mercury----------	56.85 %-	61.53 %

In the determination of the sulphide,it was difficult to decompose all the suspended double salt. Some of the particles were found to be still unchanged when transfered to the filter. Hence,this fact would account for the lower percentage of the sul-

phide and consequently of the mercury in our salt. Making this correction,it is quite evident that this salt is the same compound as that of Johnston's. It was studied further,however,as to its solubility and found to be as follows: insoluble in 90% alcohol,ether,ligrion and benzol;slightly soluble in 95% alcohol, chloroform and sulphuric acid; medium soluble in absolute alcohol,carbon-di-sulphide and amyl alcohol,and quite soluble in aniline,hydrochloric and nitric acids. The salt contained no sulphates or phosphates. The quantity of the first salt as compared with the second was about twice as great.

The second salt contained:

Nitrogen---------- 0.131 %

Mercuric sulphide---91.420 %

Mercury----------78.800 %

This was insoluble in alcohol,ether,benzene,chloroform,carbon-di-sulphide,ligrior,amyl alcohol and sulphuric acid;slightly soluble in nitric and hydrochloric acids,and readily soluble in aniline and a mixture of aqua regia and potassium chlorate on heating. It will be seen that the two salts are very different in all respects. The second seems to be mainly an inorganic salt of mercury,perhaps the chloride itself.

By treatment with mercuric chloride,the greatest portion of the creatinine was hoped to have been removed. The excess of the mercury was gotten rid of by means of hydrogen sulphide,and

the nitrogen determined on the precipitate. It was found as supposed,that very little nitrogen was carried down mechanically with the sulphide precipitate. The filtrate was now heated on the water bath,in order to drive off all the excess of hydrogen sulphide. Upon evaporization more of the salt came down. The solution was refiltered and the filtrate made up to a definite volume. It was analyzed. The results are comparable with the last analyses.

Total Solids----------	131.14 grs.----------	
Nitrogen as flesh bases--	29.09 grs.-22.18 %----	13.96 %less.
Ash----------	15.00 grs.-11.44 %----	1.91% less.
Other substances---------	87.06 grs.-66.39 %----	10.72 %more.

In this determination,the weights of the total solids were very difficult to obtain constant. The hydrochloric acid,set free by the hydrogen sulphide,seemed to be the cause and thus introduced an error which had to be removed.

The next question,which arose,was how to separate out the creatin,still remaining in the solution. Engel's method (41) was tried:adding an excess of the precipitating reagent,then enough caustic potash to make the solution alkaline. In this way, he claimed that the creatin was changed to creatinine,after which it easily came down as a double salt. It was found that the reaction was not as complete and as well accomplished as the fol-

lowing:change all the creatin into creatinine by heating the strongly acidified solution for several hours. Then add either mercuric chloride as before or freshly precipitated mercuric oxide. The latter was used,since it accomplished a two fold object;the removal of much of the hydrochloric acid and also that of the flesh-base body. The filtrate from the sulphide was boiled for three hours with about thirty percent of its solution composed of dilute hydrochloric acid. While hot,the moist oxide was gradually added. The liquid was vigorously stirred after each addition. At first,the material was completely dissolved but soon a precipitate,very similar to the former double chloride salt,came down. When the solution was neutral to litmus,noting the fact that the chloride of mercury has a slight acid reaction,no more oxide was added. The mixture was then allowed to stand over night,when it was filtered and the precipitate thouroughly washed with a dilute solution of mercuric chloride. The filtrate,together with the washings,were evaporated to a small volume and the second mercury salt came down as before. This was filtered off and the excess of mercury removed by hydrogen sulphide. The quantity of this chloride salt was very large and probably contained some of the unchanged mercuric oxide. However, it was not analyzed. The clear filtrate from this treatment was much lighter in color,but upon heating it,the darker shade reappeared,showing that the change in color was probably due to

dilution. The analysis of the solution is as follows,the results being comparable with the previous filtrates:

Total Solids----------74.57 grs.....

Nitrogen as flesh-bases--11.73 grs.--- 15.73 %-----6.35 %less

Ash----------6.96 grs.--- 9.33 %-----2.11 %less

Other substances by diff.55.88 " .--- 74.93 %-----7.54 %more.

The solids,upon evaporization.came down in much better shape than in the last case. The amount of chlorine given off was also much less. The weight of the solids became constant sooner but it was noticed that during the last heating,decomposition was commencing to take place.

The next step was to remove the remaining hydrochloric acid and,if possible,more of the nitrogen. To do this,a reagent which would react as the mercuric oxide,in a double manner,was sought. According to Engle (42),silver nitrate precipitates creatine,hence it was decided that the oxide would do the same thing and also remove the chloride. To an aliquot portion of the warmed filtrate,from the last treatment,moist freshly precipitated silver oxide was carefully added,while stirring. At first a portion went into solution but soon a flocculent,greyish precipitate formed. After adding quite a considerable of the material, the solution was tested with litmus. It was hoped that an almost neutral state might be reached,prefering to have it slight-

ly alkaline. Thus insuring that all the hydrochloric acid had been taken up, When the neutral point was about reached,a white precipitate came down. It was very much the same as silver chloride, which it probably was. When the desired point was reached,the temperature increased quite preceptibly and a fishy odor was given off which was,no doubt,due to amines of some sort. The solution was allowed to stand and upon filtering it was found that washing the precipitate,the filtrate became very turbid. However,it was refiltered several times and analyzed. Upon heating,the total solids gave evidence of decomposition and a separation even took place in the solution in the flask at room temperature. Hence,it was thought advisable not to continue in this direction. The above process was repeated,but after reaching the alkaline state, the solution was titrated back with some of the same material until a slight acid reaction was reached. In this way,it was thought that the filtration would perhaps be better and the escape of amines and other substances would be prevented. From the amount of silver oxide required,it was quite evident that a portion of it, went to form soluble salts with some of the organic acids. It was also apparent that at the same time an opposite reaction was going on,as the appearance of the insoluble residue was very materially changed from that of the oxide itself. Upon filtering, there was no trouble in washing the precipitate and in obtaining a clear filtrate. This was made up to definite volume and analyzed:

Total Solids	77.28grs.		
Ash	23.33 "	30.18%	20.75% more.
Nitrogen as flesh bases	9.42 "	12.18%	3.55% less.
Other substances	44.53 "	57.75%	17.18% less.

From the above analysis,it will be seen that the ash content has been increased over twenty per cent. The only explanation for this is,as previously stated,that some of the silver oxide formed soluble salts and consequently the silver in solution increased the mineral matter. It will also be noticed,that the percentage of non-nitrogenous material has lowered. This is due partly to the high ash increasing the total solids and hence lessening its per cent,and no doubt also to the fact that the oxide carried down some of this material mechanically or chemically. However,the object was accomplished as regards the removal of hydrochloric acid and more of the nitrogen. The question remained to be proven as to whether it was the wise thing to introduce this last reagent at the expense of the increase in ash.

It was now beleived that all the creatinine that was possible by means of salts,had been removed and if there was any of it remaining,some other method would have to be brought into use. It now began to be apparent that the mineral matter would have to be gotten rid of,but at the same time,the sole object in the work was to retain as much of the non-nitrogenous material as possible,

and not to treat the solution in any way which would reduce that part. The filtrate from the last treatment was now evaporated to a syrupy consistency and allowed to stand for several days in a vacuum dessicator. By this means,it was thought that the evaporated solution might crystallize and then the crystals could be purified by recrystallization after which one form could be separated from the other. In this way,the mineral matter could at least be removed. After several days,a few crystals began to form. There were two kinds,the one needle-like and the other similar to those of creatin and creatinine. But the main portion of the material remained in the same syrupy condition. It was finally decided that some other treatment would have to be resorted to.

The fact that peptones,creatin,creatinine,and potassium salts are insoluble in absolute alcohol led to the use of it upon this residue. It was thought that these constituents might not only be gotten rid of,but that at the same time a filtrate,which would crystallize might be obtained and thus obtained in a condition to purify. Upon adding absolute alcohol to a portion,a milky white effect was at first produced,then a white flocculent precipitate came down but upon adding an excess of the alcohol, it seemed to dissolve the precipitate and render it sticky and darker in color. The alcoholic liquid was decanted and the precipitate examined. Part of it was dissolved in a little water and then alcohol again added. This time,the precipitate came down

granular but upon exposure to the air,it returned to its syrupy condition. The remainder of the precipitate was tested as to whether it was a base or not. It was found to require considerable acid to overcome its basic properties. From this,it was at first concluded that the material was a base,but it was finally decided that this property was due to the sodium and potassium oxides formed in removal the chloride with the silver reagent. When the acid state was reached the precipitate became granular and formed well defined crystals. This crystalline precipitate was transfered to a watch glass and dried over sulphuric acid. It was thought that perhaps creatinine or creatin might cause this reaction and a test was made upon a sample of the pure product. No such reaction took place. However,nitrogen determinations were made upon the salt and the pure product. In this manner,both the method and the amounts of nitrogen in each were tested. There are some substances which do not give up their entire amount of nitrogen by the Kjeldahl process and it was thought that perhaps creatin might be one of them. There was no nitrogen in the salt, while the pure product contained 27.41 % which agreed quite closely with the theoretical amount in that our product may not have been all creatin but partly creatinine-chloride. From this,it was concluded that the two salts were not alike,however the former was analyzed further. It contains 6.7% of organic matter. Of the inorganic portion,the greater part was composed of sodium and potassium. Hence the salt was almost entirely inorganic contain-

ing a little non-nitrogenous material.

It was now apparent that much of the mineral matter had been removed in this way and it was hoped that the remainder of the substance would crystallize. This liquid was first analyzed and then the rest of it was evaporated to dryness and put in a dessicator.

Total Solids----------	71.95 grs.----------		
Ash----------	21.40 "	.-29.74%-----	0.44% less.
Nitrogen as flesh-bases-	7.32 "	.-10.17%-----	2.01% less.
Other substances by difference----------	43.13 "	.-59.95%------	2.20% more.

After forty-eight hours, no crystals were found to be present and the analysis was continued.

If there was any mineral matter present in this evaporated residue, it ought not to be soluble in absolute alcohol. Upon extracting a portion of it with the smallest possible amount of the alcohol, nothing was left undissolved. From this it was evident that the mineral matter, which was there, was a peculiar kind. Another portion of the same material was taken and dissolved in a small amount of absolute alcohol and then an equal quantity of ether was added. A cloudy effect was produced and on cooling the ether layer was colored, showing that some material had been extracted in this process. Again, some of this residue was treated by satur-

ating its water solution with bromine. It was thought that in this way,perhaps the substance in question might form a substitution product and then crystallize. The bromine was added gradually and the amount required was much greater than that needed to saturate the same quantity of water. When complete,the liquid was extracted repeatedly with ether. All the color was removed thereby. The extract was evaporated to a small volume and allowed to stand for a couple of days. A few crystals formed,but the main portion was still a sticky mass. In that part,which was not extracted with ether,quite well defined crystals formed. These were washed with absolute alcohol and found to be slightly soluble in the media. They were then dissolved in water and the solution evaporated almost to dryness. Crystals formed a ain. Qualitative tests of them showed the presence of sodium,potassium and a very small amount of organic matter. Another treatment was to dissolve some of the substance in glacial acetic acid and allow the solution to evaporate. Some crystals were formed,but the greater portion was apparently unchanged. Absolute alcohol was gradually added to this acid residue. A heavy granular precipitate immediately formed. Upon transfering it to a filter and bringing it in contact with the air,the precipitate returned to its sticky condition. Analysis of it showed,however,that there was very little mineral matter present.

In all these attempts to crystallize the material none of

them seemed to be of much value. The small amount that did crystallize proved to be mostly mineral matter. At this point,it was thought that the substance which was interfering in the crystallization might be glycerine,caused,perhaps,by the saponification of some of the acids. Tests were made to determine its presence or absence. At the same time,other tests were made. It was conclusively proven that no glycerin was present. Alkaloid reagents were tried. Wagner's,Mäyer's,Tannic acid,and Sonnenschien's all gave amorphous precipitates. Picric acid gave none. Fehling's solution, used for reducing substances,produced no copper oxide precipitate but the blue color was changed to a greenish one. Heller's test for albumen,gave no reaction. Million's reagent proved the absence of proteids. From this,it is apparent that the nitrogenous body is not a proteid,a reducing substance or glycerin. The indications seem to point to some substance having alkalidal properties.

After making all these tests upon this non-crystallizable material,it was decided to treat the entire amount with absolute alcohol and ether. If the mineral matter was to be gotten rid of there seemed to be but this one method left for its accomplishment. Nearly,if not all,inorganic salts are insoluble in the one or other of these reagent or in their mixture. Creatin,creatinine, all peptones,and their decomposition products,and the known reducing substances are also insoluble in these media. Hence,by proper use of them,the remainder of these ingredients ought to

have been removed. However,care had to be taken not to go beyond the boundary line and take out,at the same time,the material which was being isolated. On this account,it was deemed advisable to extract the residue by fractionalization:first using a mixture with the alcohol in greater proportion than the ether then,after each step,decreasing the percentage of the former by gradually increasing that of the latter. By this means,it was hoped that upon analyzing the liquid after each extraction,that a point would be reached where either the extractive material or the extractive itself would contain little or no nitrogen and mineral matter,only the non-nitrogenous portion.

The remaining substance was dissolved in as small a quantity of absolute alcohol as possible,then half this amount of anhydrous ether was gradually added while shaking. An oily,dark colored liquid separated out on the bottom. After allowing the mixture to stand for about an hour,it was decanted through a dry filter and the residue washed with a known quantity of alcohol and ether,mixed in the same proportion. To the filtrate,ether of sufficient quantity to raise its percentage one fourth was added. The oily mass formed,in this case,was less in amount and lighter in color than in the previous one. It was much clearer and more transparent. The second filtrate was now extracted with equal quantities of the reagents. The amount of the oily,sticky,material was still less but its appearance was the same as that from the second treatment. Finally,a fourth extraction was made,using one fourth

more of the ether than of the alcohol. The material still continued to be precipitated but the amount was very small. After standing a few hours, the first and second residues showed signs of crystallization but it was concluded that this was due to the mineral matter removed by the process. Analysis was made of each filtrate before entering upon the next extraction. When the fourth operation had been completed, it was decided that the process had been carried far enough.

In the following table, are given the analysis of each extract. They are not comparable with any of the preceeding analyses, since an unknown amount of the material was taken for the above tests.

Analyses of Extractives.

Lab. No.	Total Solids	Ash	Nx3.12	Other sub. Diff.	Nitrogen	Nx3.12	Ash	Other Sub.
	grams	grams	grams	grams	%	%.	%	%
1186E10	20.475	1.944	3.335	15.196	3.22	16.29	9.49	73.73
1186E11	14.076	.911	2.184	10.981	4.99	15.52	6.47	78.01
1186E13	9.269	.49	2.153	6.621	7.4	23.24	5.34	71.43
1186E15	6.98	.07	1.491	5.423	3.84	21.35	1.00	77.72

From the above table it will seen that in the last extraction, 1186E 15, almost all the mineral matter was removed and that the

amount of nitrogen was decreased about one half that in the first extractive,1186F10. The fact that some of the nitrogen clung so firmly,tended to indicate that the material determined by difference and thought to be,perhaps,entirely non-nitrogenous was in fact,a substance containing a small percentage of nitrogen. If this was true,then we had,in this extract,the desired substance in almost a pure state,especially as regards mineral matter. However,it may be that this material was still composed of one or more constituents.

Time prevented a further research in the attempt at separation along this line,and it was continued in studying and endeavoring to identify the constituent or constituents in this material and that of the extracted residues. The final ether extract was evaporated to a syrupy consistency and then placed in a dessicator,together with the residues,to dry. The second,third and fourth extractives were dissolved in water and put together,then evaporated almost to dryness,after which they were disposed of as just stated. The object in analyzing both of these substances was to determine their similarities and differences. By so doing,it was thought that some clue might be obtained as to the chemical behavior of the material in question. Tests were carefully made for all the constituents that could possibly have been in the meat and for the decomposition products formed by the various treatments which it had been subjected to:proteids,albumins,peptones and their decomposition products,leucin and tyrosin;reducing sub-

stances,glucoses,glycogen,inosite;lecithens;creatinine and alkaloids. The results are tabulated below:

Ether Extractive Solution.

Reagents	Tests for.	Result of Tests.
Xantho-proteic Reaction.	Proteids.	Very slight change on heating.
Con. H_2So_4.	Proteids.	Darkens and charrs when heated.
$K_4Fe(CN)6$ + Acetic acid.	All proteids except peptones.	No action.
Picric acid.	Proteids.	No action.
Picric acid + Acetic acid.	Proteids.	No action.
Neutral lead Acetate.	Proteids.	No action.

Ether Extractive Solution (con.)

Reagents.	Tests for.	Result of tests.
Tannin +Acetic acid	Proteids.	No action.
Glacial Acetic + $H_2S\ O_4$(con.).	Proteids with peptones.	No action.
Liebermann's test.	Proteids.	No action.
Phospho-molydic acid.	Proteids creatinine& alkaloids	No action.
Ferric chloride. (Neutral).	Peptones.	No action.
$K_4Fe(CN)_6$	Peptones	No action.
Phospho-tungstic acid.	Alkaloids, Peptones & creatinine.	White,amorphous precipitate.
Tauret's Reagent.	Albumins	No action.
Million's Reagent.	Albumins.	Yellowish amorphous precipitate. Dissolves in excess.
Buiret test.	Albumen and gelatin.	No action.

Ether Extractive Solution.

Copper Sulphate solution	Albumin.	No action.
Silver nitrate solution	albuminates	White amorphus precipitate.
Mercuric chloride	albuminates and creatinine.	No action.
Basic lead Acetate.	Albuminates creatinine.	No action.
Sodium carbonate + Fehling's solution.	creatinine.	No precipitate, green color produced.
Salkowski's test.	creatinine.	No action.
Picric acid + Caustic Potash.	creatinine glucose and Acetones.	No action.
Phenyl-hydrazene hydrochloride etc.	Glucoses.	No action.
Scherer's test.	Inosite.	No action.
Scidel's test	Inosite	No action.

Ether Extractive Solution.(con)

Scherer's test.	Tyrosin	No action
Pira's test.	Tyrosin.	No action.
Scherer's test.	Leucin.	Darkened slightly with NaoH.But did not form oily mass on igniton.
Tannin	Alkaloids	No action.
Mäyer's Reagent	Alkaloids and Proteids.	White,bulky,amorphous precipitate.
Wagner's Reagent	alkaloids.	Brown,amorphous precipitate.
Platinic Chloride.	alkaloids.	No action.
Gold Chloride.	alkaloids.	No action.
Molybdic solution	Phosphates and Lecithens	. . . . .

Ether Extractive Residue.

Reagent	Test for.	Result of tests.
Xantho-proteic reaction.	Proteids.	No action.
Con. $H_2S\ C_4$.	Proteids	Darkened very slightly on heating.
K_4 Fe $(CN)_6$ + acetic acid.	all proteids except peptones.	No action.
Picric Acid.	Proteids.	No action.
Picric acid + acetic acid.	Proteids.	No action.
Neutral lead acetate.	Proteids.	No action.
Tannin + acetic acid.	Proteids and Peptones.	No action.
Glacial Acetic + con. H_2SO_4.	Proteids and Peptones.	No action.

Ether Extractive Residue.(con)

Reagent.	Tests for.	Result of tests.
Liebermann's test.	Proteids.	No action.
Phospho-molydic acid.	Proteids and creatinine and alkaloids.	Light brown amorphous precipitate.
Ferric chloride (neutral)	Peptones.	No action.
$K_4Fe(Cn)_6$.	Peptones.	No action.
Phospho-tungstic acid.	Peptones alkaloids and creatinine.	White,amorphous precipitate.
Tauret's Reagent.	alkaloids and albumins.	White,amorphous precipitate.
Million's Reagent	Albumins.	No action.
Biuret test.	albumina and gelatin	No action
Copper Sulphate Solution.	Albumin.	No action.
Silver Nitrate.	Albuminates.	White,amorphous precipitate.

Ether Extractive Residue.(con).

Reagents.	Tests for	Results of tests.
Basic lead acetate:	albuminates and creatinine.	No action.
Mercuric chloride.:	albuminates and creatinine.	No action.
Sodium Carbonate + Fehling's solution:	Creatinine.	No action.
Salkowski's test.:	Creatinine.	No action.
Picric acid + Caustic Potash.	Creatinine Glucose and Acetones.	No action.
Phenyl-hydrazine hydrochloride etc.	Glucoses.	No action.
Scherer's test.	Inosite	No action.
Seidel's test.	Inosite	No action.
Scherer's test	Tyrosin	No action.
Pira's test	Tyrosin	No action.

Ether Residue Extractive (con)

Reagents.	Tests for	Results of tests
Scherer's test	Leucin	Darkened slightly with NaOH.But did not form oily mass in ignition.
Tannin	alkaloids.	slight yellowish amorphous precipitate.
Mayer's Reagent	Alkaloids and Proteids.	No action.
Wagner's Reagent.	alkaloids.	No action.
Platinic chloride	alkaloids.	No action.
Gold chloride	alkaloids	No action.
Molybdic solution	Phosphates and Lecithins.	.....

From these tables,it will be seen that the evaporated ether solution gave good tests for alkaloidal substances. There was a slight indication of the presence of leucin but it was not complete enough to give a positive proof. The silver nitrate precipitate might have been due to the presence of chlorides. The ether extractive residue gave results agreeing quite closely with those made upon the ether solution. There was more evidence of alkaloidal properties,but a-

side from this no other variations were noticed. By these tests, the two substances were found to be practically identified and free from all constituents that could, as far as present knowledge upon the subject extends, be there.

As a final step in this work, these residues were completely analyzed. Determinations were made for ash, organic matter, nitrogen by Kjeldahl process, and carbon and hydrogen by combustion. In this last case, modification was made for the nitrogen present in the bodies. By this means, data was obtained whereby formulae could be calculated for the organic material in each substance. Having done this, each was studied by comparing the formulae as to structure, composition, similarity and identity. The result of the analysis of the ether extractive solution is as follows:

Ash	4.93 %
Organic matter	95.07 %
Nitrogen calculated to organic portion	4.91 %
Carbon " " " "	45.04 %
Hydrogen " " " "	7.35 %
Oxygen " " " "	37.77 %

The formula calculated to the organic material is $C_{11}H_{21}NO_6$.

For the ether extractive residue, the ash and nitrogen was found to be high. The combustion was unsatisfactory as the mineral matter, upon fusing, seemed to inclose some of the carbon. However

the formula was calculated and found to be $C_{10}H_{23}NO_2$..

From this,it appears that the two substances are not alike although no positive statement can be made regarding the latter because of its incomplete combustion. Until a better method can be devised for correcting the error ,nothing definite can be said.

In conclusion,a brief review of this work will be made with a few suggestions for the modification of the method. It was found best not to evaporate the ninety-five per cent alcohol filtrate to dryness but to a small volume. In this condition,crystals of creatin and creatinine will be able to separate upon standing and thus aid much in the removal of nitrogen. By using small quantities,a much less percentage of the substances determined by difference is obtainable. The bulky precipitate formed,seems to carry down mechanically a considerable of the material with it. Of all the reagents used for the removal of the foreign ingredients,mercuric chloride removed the most nitrogen and the oxide,the most mineral matter. However,for completeness and thoroughness,the mixture of four parts of absolute alcohol and five of anhydrous ether served admirably. The following table gives a resume of the analysis of each step taken as far as the use of the alcohol and ether,where the results are not comparable and hence not included here.

Filtrate after treatment with	Total Solids	Ash	Nitrogen x3.12	Other Subs. Det.by Diff.	Per cent removed calculated to original.		
					Ash	Nx3.12	Other Sub.
	grams	grams	grams	grams	%	%	%
Ninty-five per cent alcohol.	229.76	30.68	83.02	104.94	----	----	----
Barium Hydroxide.	226.16	27.80	82.98	115.38	-1.26	-0.03	+4.55
Mercuric Chloride.	125.23	19.29	28.04	77.90	-3.70	-23.87	-16.31
Mercuric Oxide	74.57	6.96	10.78	56.83	-5.37	-7.11	-9.18
Silver Oxide	77.28	23.33	9.42	44.53	+7.13	-1.02	-5.35
Absolute alcohol.	71.95	21.40	7.32	43.13	-0.84	- .90	-0.60

It can be seen from this, that the first alcoholic filtrate contained, as previously suggested, more creatinine than creatine, as the per cent of nitrogen removed by the mercuric chloride is greater than the amount precipitated by mercuric oxide, after having converted all the creatin into creatinine. It also appears that in as much as the filtrate from the silver oxide treatment is to be extracted with absolute alcohol and ether together the use of the former alone is unnecessary. It is quite probable that the expensive silver oxide can be replaced by lead carbonate but the carbon

dioxide set free by its use might cause some serious decomposition of the material. In the determination of mineral matter more accurate results would have been obtained if the official method had been used, It was found,however,that the loss due to the volitization of sodium and potassium chloride was so small that it need not enter into the argument as being the cause of the deficiency in obtaining the summation of the constituents of beef broth.

Finally,it must be stated that the investigation upon this subject is far from complete. There are,no doubt,other modifications to be made upon this method,other theories of explaining the deficiency,other channels of approaching this problem,other means of continuing the separation and other tests to be applied for identification,yet,basing the statement upon what has been done thus far,it can safely be said that the error in the analysis of beef broth is due,at least in part,to the presence of non-crystallizable organic matter of alkaloidal properties containing a small percentage of nitrogen.

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May 30th, 1901.

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